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Application of scanning electrochemical microscopy to biological samples

(profilometry/topography/voltammetry)

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ABSTRACT **The scanning electrochemical microscope can be used in the feedback mode in two-dimensional scans over biological substrates to obtain topographic information at the micrometer level. In this mode, the effect of distance between a substrate (either conductive or insulating) and a scanning ultramicroelectrode tip on the electrolytic current flowing at the tip is recorded as a function of the tip *x–y* position. Scans of the upper surface of a grass leaf and the lower surface of a *Ligustrum sinensis* leaf (which show open stomata structures) immersed in aqueous solution are shown. Scans of the upper surface of an elodea leaf in the dark and under irradiation, where the tip reaction is the reduction of oxygen produced by photosynthesis, demonstrate the possibility of obtaining information about the distribution of reaction sites on the substrate surface.**

The scanning tunneling microscope (STM) (1) has led to several variants, such as the atomic force microscope (2) and the ion-conductance microscope (ref. 3 and refs. therein to other types of scanning microscopes). These latter devices are useful in examining the topography of electrically insulating substrates that cannot be studied with the STM. Previous studies from this laboratory have described the scanning electrochemical microscope (SECM), the theory of the feedback mode, and several applications (4–7). In microscopy with the SECM, an ultramicroelectrode (UME), with a tip radius of the order of μm or less, is moved in close proximity to a substrate of interest that is immersed in a solution containing an electroactive species (Fig. 1). The electrode reaction at the tip gives rise to a tip current that is affected by the presence of the substrate. In general, the steady-state tip current, i_T, is controlled by electrochemical reactions at the tip electrode and is a function of tip–substrate distance, d, and the conductivity and chemical nature of the sample substrate. The measurement of i_T can thus provide information about topography of the sample surface (6, 7) as well as its electrical and chemical properties. In the feedback mode, the magnitude of i_T increases with respect to its steady-state value at large distances from the substrate ($i_{T,\infty}$) when the tip electrode is moved close to a conductive substrate and decreases when the tip electrode is moved close to an insulating substrate (6). Note that this feedback mode is different than the SECM generation/collection mode (1, 8, 9), which requires a conductive or semiconductive sample. In this paper, we demonstrate the first application of the SECM to *x–y* (topographic) scans of biological substrates—e.g., leaves of grass, ligustrinum, and elodea.

EXPERIMENTAL

Instrumental details for SECM have been described (6). Milli-Q reagent water (Millipore) was used for the aqueous solutions, with dissolved $K_4Fe(CN)_6$, $Ru(NH_3)_6Cl_3$, K_2SO_4, and/or KCl, as received. A platinum microdisk electrode tip (radius, 1.0 μm) and a carbon microdisk electrode tip (radius, 5.5 μm) were fabricated as described (4, 10). A 250-W tungsten-halogen lamp (model SFL-135-102, lamp type 250-T40-CL, Sylvania Electric Products, Fall River, MA) was used as a light source.

RESULTS AND DISCUSSION

Upper Surface of a Grass Leaf. Topographic images of a section on the top surface of a blade of grass taken with a SECM are shown in Fig. 2 (*Upper*). The grass was immersed in an aqueous solution of 20 mM $K_4Fe(CN)_6$ and 0.1 M KCl, and the UME tip, a 1-μm radius Pt disk UME held at +0.7 V vs. a saturated calomel reference electrode (SCE), was brought near the grass substrate and scanned over its surface. At the applied potential, the electrode reaction at the tip, $Fe(CN)_6^{4-} - e \rightarrow Fe(CN)_6^{3-}$, causes an anodic current. When the distance between tip and substrate, d, is large compared to the UME radius, a, i_T is independent of d ($i_T = i_{T,\infty}$). When the tip is moved closer to the sample ($d \leq 4a$), i_T becomes smaller than the long distance value. Since $i_T < i_{T,\infty}$, the surface is an electronic insulator. The extent of the decrease of i_T from $i_{T,\infty}$ ($\approx$2.63 nA) is a measure of the tip–substrate distance, d; the closer the tip is to the substrate, the smaller is i_T, because the substrate blocks diffusion of $Fe(CN)_6^{4-}$ to the tip. The magnitude of $i_T/i_{T,\infty}$ can be used to determine a/d (5, 6). Thus the SECM scan, unlike an optical microscope, probes the surface topography of the sample.

The variation of i_T can be converted to a color intensity to produce the gray-scale presentation, taken over an area of 188.3 × 141.7 μm with the tip scanned at 23.7 μm/s above the substrate, as shown in Fig. 2 (*Upper*). In Fig. 2 the white lines around the edge denote a 10-μm distance, and the scale at the left shows the gray-scale variation: dark color, maximum anodic current and large d; light color, minimum anodic current and small d. The average size of each cell in the grass blade was $\approx$30 μm. Fig. 2 (*Upper*) also shows the parallel venation pattern characteristic of monocot leaves (11).

Lower Surface of a *Ligustrum sinensis* Leaf. SECM scans over the bottom surface of a *L. sinensis* leaf immersed in an aqueous 20 mM $K_4Fe(CN)_6$/0.1 M KCl solution are shown in Fig. 2 (*Middle* and *Lower*) as gray-scale images. The tip scan speed, the scanned area, and tip were the same as those used for Fig. 2 (*Upper*). In Fig. 2 (*Middle* and *Lower*), the maximum anodic tip currents were 2.75 and 2.32 nA, and the minimum anodic tip currents were 0.73 and 0.98 nA, respectively. Several open stomata structures are shown clearly in these figures. Stomata were usually much more abundant on

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Abbreviations: STM, scanning tunneling microscope; SECM, scanning electrochemical microscope; UME, ultramicroelectrode; SCE, saturated calomel electrode; CV, cyclic voltammetry.

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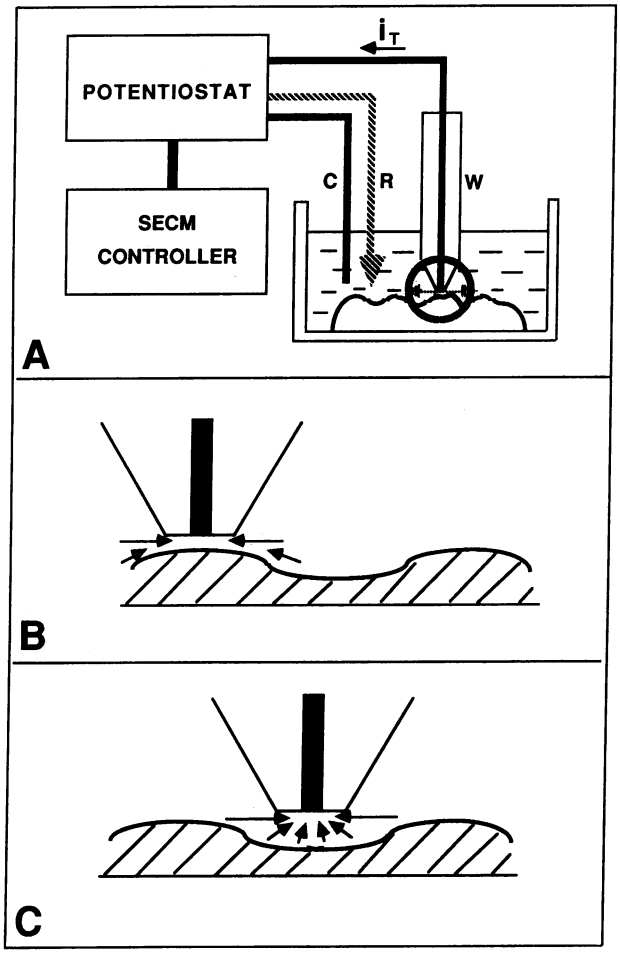

FIG. 1. (*A*) Schematic diagram of SECM scanning tip above a substrate. W, C, and R, tip, counter, and reference electrodes, respectively. (*B* and *C*) Enlargement of circled area in *A* for insulating substrate. (*B*) More hindered diffusion at small *d* (smaller i_T). (*C*) Less hindered diffusion at large *d* (larger i_T).

the bottom surface of the leaves (11). Each stoma is surrounded by a pair of specialized epidermal cells called guard cells. In open stomata, these guard cells are swollen and protrude above the surrounding epidermal cells seen in the gray-scale topography. In SECM scans near an insulating substrate surface, the relative response of i_T at small *d* (above the raised areas) is much more sensitive than that of i_T at large *d* (above the indented areas) (5). This effect accentuates the contrast of the protruding guard cell with respect to other lower areas, and i_T representations do not yield the actual size scale directly (as is also true in other related microscopies, such as STM). However i_T can be related to *d* via known relationships (5).

Upper Surface of an Elodea Leaf. The scanning tip can also be used to detect electrochemically products generated at a biological substrate—for example, as widely used in nontopographic studies of the production of neurotransmitters in cells (12) or to determine, in the feedback mode, how tip generated species interact chemically with the biological substrate. To illustrate this operating mode, we report preliminary studies of the detection of oxygen during illumination of a leaf of elodea, a well-known water plant that is often used to demonstrate oxygen evolution in photosynthesis. A disk-shaped carbon-fiber UME (radius, 5.5 μm) was used as the tip to detect oxygen reduction rather than a Pt UME, to avoid interference by proton reduction at the tip potentials used. The mechanism of oxygen reduction at a C electrode is complex and is strongly dependent on the mode of pretreatment of the

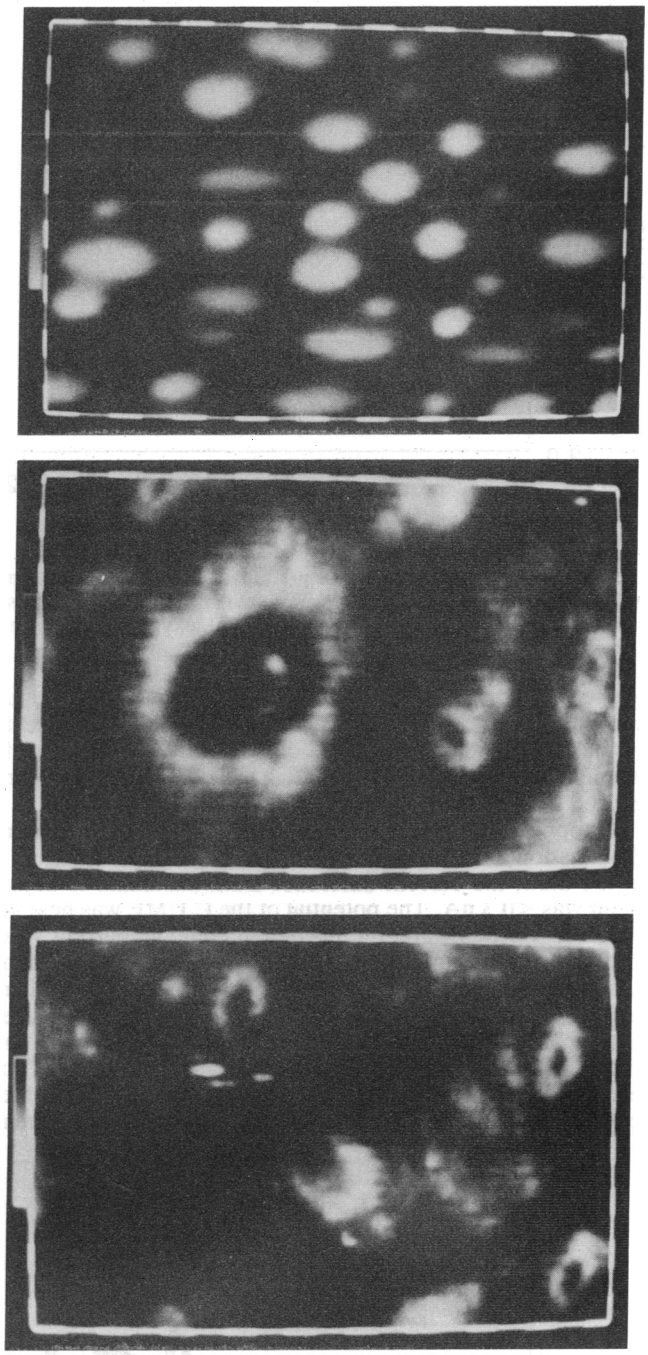

FIG. 2. Gray-scaled image of biological substrates in a $K_4Fe(CN)_6$/KCl solution scanned with Pt UME (radius, 1 μm). (*Upper*) Top surface of grass. (*Middle* and *Lower*) Bottom surface of *L. sinensis* leaf. Small openings are stomata.

C surface (13, 14). In the study reported here, the C UME was polished with 0.05 μm alumina, and cyclic voltammetry (CV) of a 10 mM KCl solution presaturated with N_2, air, and O_2 was examined to determine the UME potential for O_2 reduction (Fig. 3). These results are in agreement with those previously reported for O_2 reduction at C. To examine oxygen evolution under illumination, elodea leaves were immersed in a 10 mM KCl solution presaturated with CO_2. The C UME tip was positioned 200 μm above the leaf surface and CV was carried out at v = 200 mV/s in the dark (Fig. 4, curve a), under illumination (250-W tungsten-halogen lamp) (Fig. 4, curves b and c), and then again in the dark (Fig. 4, curve d). Fig. 4 depicts changes in local concentration of substrate-generated

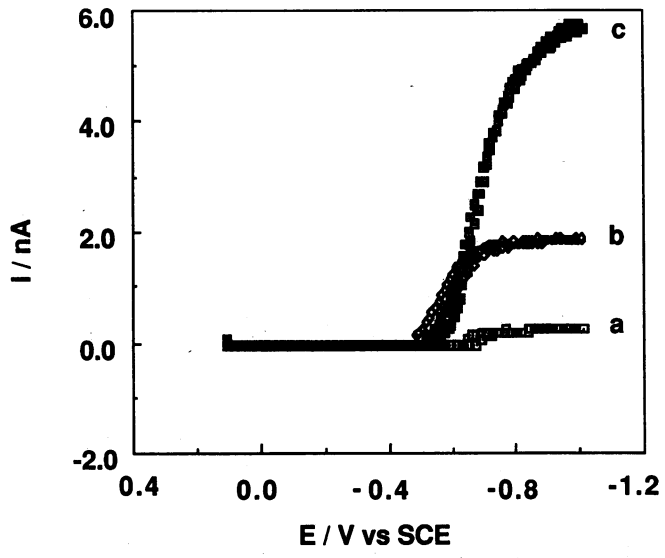

FIG. 3. Cyclic voltammetry of pretreated 10 mM KCl solution with a disk-shaped C UME (radius, 5.5 μm; v = 50 mV/s). Solutions saturated with nitrogen (curve a), air (curve b), oxygen (curve c) for 2 min.

oxygen by photosynthesis. If the same experiments were carried out with chloroplast-deficient leaves, the enhancement of the O_2 cathodic current was only 0.41 nA after 6 min of illumination. The chloroplast content of elodea leaves could be gauged by optical microscopy.

A topographic (x–y) scan could also be obtained under illumination in a CO_2-saturated 10 mM KCl solution (Fig. 5); in the dark, the current difference from maximum to minimum was <0.3 nA. The potential of the C UME was held at −0.85 V vs. SCE, and the maximum cathodic current was 1.89 nA and the minimum current was 0.49 nA. The area scanned and the tip scan speed were the same as in the other experiments. Basically, the topography of the upper surface of the elodea leaf is similar to that of the grass leaf except for the sizes of the cells (≈50 μm). Optical microscopy of an elodea leaf shows small stomata located sparsely within the cell, which are divided by thicker cell walls. Molecular

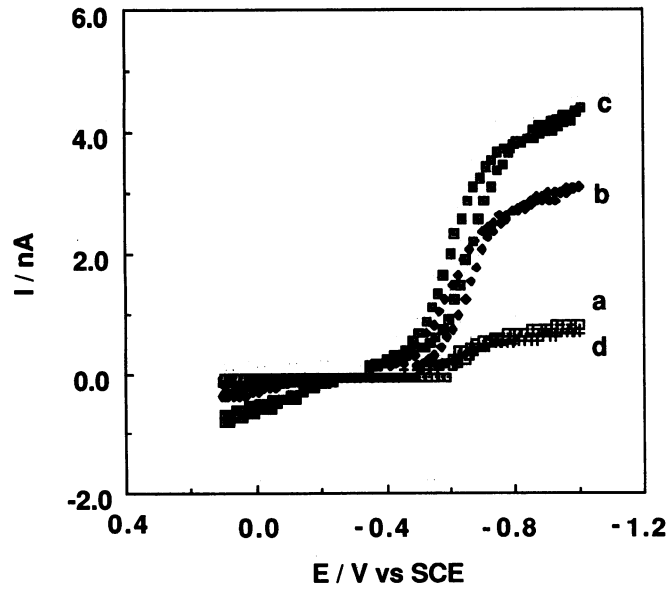

FIG. 4. Change of CV while illuminating with a 250-W tungsten halogen lamp for a C UME held 200 μm above an elodea leaf: initial, in dark (curve a), after illuminating 6 min (curve b), after illuminating 10 min (curve c), and 12 min after curve c in the dark (curve d).

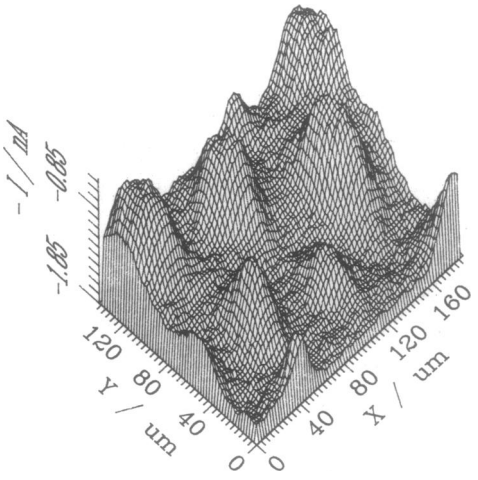

FIG. 5. Topographic (x, y) scan of the top surface of an elodea leaf in CO_2-rich 10 mM KCl solution under illumination.

oxygen is secreted through the stomata, which are distributed sufficiently sparsely to show spatial differences in the enhancement of local oxygen concentration (Fig. 5). A similar topography was obtained with the elodea leaf in the dark immersed in 10 mM $Ru(NH_3)_6Cl_3$/0.1 M K_2SO_4 solution.

CONCLUSIONS

The results presented here clearly demonstrate the application of the SECM to obtain three-dimensional scans and topographic information about biological samples immersed in an electrolyte solution by using the feedback mode or a substrate-generated electroactive species (oxygen). Although absolute height scales were not determined in the topographic scans presented, these are obtainable through straightforward transformation of i_T to distance. An alternative approach would involve the use of a constant i_T mode, as frequently used in STM, where topographic information is obtained from the voltage applied to the z-piezo axis to maintain a given value of i_T. This mode would only be useful, however, for substrates that do not have both conductive and insulating regions. Chemical identification of zones on a substrate appears to be a potentially important application of SECM. For example, generation of a species at the tip that could react at appropriate sites on the substrate (e.g., those containing an enzyme) would provide information about site distribution, size, and shape. Similarly, the tip could be used to detect the distribution of neurotransmitters that are released by an appropriate stimulus, following the well-developed methods used in electrochemical studies of these systems (12, 15). Suitable electrode reactions or scanning tip designs for protons and many other species should also be available. The ultimate resolution attainable in these studies is mainly determined by the tip size. Finally, we should note that while living samples can be examined by SECM, they must be immersed in a medium in which a suitable electrode reaction can be carried out. This may sometimes lead to changes in the structure and behavior of the samples.

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